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Abstract

Glucocorticoids are illegally used as growth promoters in cattle and the analytical methods officially applied most likely underestimate the precise frequency of the abuse. As a side effect, the administration of glucocorticoids causes fat infiltration, apoptosis and atrophy of the thymus. However, gross and histological observations carried out previously showed that the thymus preserves an intrinsic ability to regenerate. The aim of this work was to study the transcriptional effects of glucocorticoids on genes likely involved in regeneration of the epithelial cell network in cervical and thoracic thymus of beef cattle treated with dexamethasone or prednisolone in comparison with the control group. Moreover, the ratio of BAX/BCL2 genes was examined to verify a possible anti-apoptotic activity occurring at the same time. In cervical thymus, dexamethasone administration increased the gene expression of c-Myc ($P < 0.01$), TCF3 ($P < 0.05$), TP63 ($P < 0.01$) and KRT5 ($P < 0.01$). In thoracic thymus of dexamethasone-treated cattle the gene expression of TCF3 ($P < 0.01$), TP63 ($P < 0.01$) and KRT5 ($P < 0.05$) was increased. These results suggested that thymic regeneration is underway in the dexamethasone-treated animals. However, the BAX/BCL2 ratio was decreased in both cervical and thoracic thymus of dexamethsone-treated cattle ($P < 0.01$ and $P < 0.05$, respectively), showing an anti-apoptotic effect through the mitochondrial pathway. Conversely, prednisolone administration caused no change in the expression of all considered genes. These results sustain the hypothesis that regeneration occurs in the thymus parenchyma 6 days after the dexamethasone treatment was discontinued. This hypothesis is also supported by the absence of alterations in thymus of prednisolone-treated beef cattle. Indeed, previous studies showed the inability of prednisolone to induce macro- and microscopical lesions in the thymus. Therefore, in this context, it is not surprising that prednisolone induced no alteration of genes involved in regeneration pathway.

Keywords: beef cattle;dexamethasone; glucocorticoids; prednisolone; regeneration; thymus

1. Introduction

The administration of natural and synthetic hormones as growth promoters in animals is banned in the European Union (EU) and analytical methods are officially applied by national surveillance programs to prevent the illegal use of hormones [1].

Dexamethasone (DEX) is one of the most commonly administered glucocorticoids (GCs), and induces fat infiltration, increases apoptosis and causes atrophy of the thymus in cattle, as a side effect [2-4]. Conversely, prednisolone (PRD), another illicitly used GC, seems to be unable to induce thymus atrophy [5]. However, it is conceivable that the thymus preserves an intrinsic ability to regenerate after GCs administration, because the bovine thymic parenchyma and activity could be restored, as previously shown by gross and histological observations [4]. Nevertheless, the mechanisms controlling thymus regeneration remain largely unknown. Conversely, no cellular response seems to be triggered by PRD administration. It was previously shown that some transcription factors are over-expressed in the thymic stroma of mice [6]. The thymic epithelial cells (TECs) showed an up-regulation of c-Myc, TCF3 and TP63 genes during DEX- or irradiation-induced atrophy and a down-regulation after regeneration. These transcription factors were previously shown to regulate differentiation of epithelial stem cells in various tissues [7-9], suggesting a role in reconstruction/maintenance of the epithelial cell network. Moreover, it has been demonstrated that DEX- and irradiation-induced damage of the thymus resulted in proliferation of specific subset of TEC precursors expressing keratin 5 (KRT5) [6].

Nevertheless, tissue re-growth is not only the result of cell proliferation, but also of enhanced cell survival by means of the inhibition of apoptosis or a combination of both mechanisms [10]. Several studies have highlighted that many of the molecular pathways involved in thymus atrophy rely on the mitochondria-dependent apoptotic pathway, involving proteins of the BCL2 family [11,12]. The members of BCL2 family are known to

67 be key regulatory proteins in apoptotic events, and can promote either cell survival or cell
68 death. Indeed, the equilibrium between the pro- and anti-apoptotic members or their
69 relative amount are crucial to sensitise the cells towards either survival or apoptosis. The
70 anti-apoptotic effect of BCL2 acts by binding and inhibiting pro-apoptotic proteins like BAX.
71 The latter promotes apoptosis by altering mitochondrial functions and activating the
72 release of downstream apoptogenic factors [13]. The aim of this work was to study the
73 biological mechanisms involved in regeneration following GCs treatment.
74 Therefore, an increase of transcript abundance of c-Myc, TCF3, TP63, and KRT5 was
75 hypothesized in the cervical and thoracic thymus during regeneration of the thymus in beef cattle.
76 Additionally, the BAX and BCL2 expression and their ratio were examined to evaluate a
77 possible anti-apoptotic activity occurring at the same time.
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80 **2. Material and methods**
81 *2.1. Animals*
82 The experiment was authorized by the Italian Ministry of Health and the Ethics Committee
83 of the University of Turin. The carcasses of the treated animals were appropriately
84 destroyed (2003/74/CE–DL 16 March 2006, No. 158).
85 All groups of experimental animals were kept in separate pens of 10 × 15 m and were fed
86 a diet consisting of corn silage, corn, hay and a commercial protein supplement; animals
87 had *ad libitum* access to water. Eighteen male Charolaise beef cattle of 17 to 22 mo of age
88 were divided into the following three groups: group A (n = 6) was administered
89 dexamethasone–21–sodium-phosphate 0.7 mg/d *per os* for 40 d; group B (n = 6) was
90 administered PRD 15 mg/d *per os* for 30 d; group K (n = 6) served as a control. Each
91 morning, before the distribution of feed, the animals were tied to the feed trough, and two
92 trained technicians administered orally one capsule containing the compound using a

drenching gun. The control animals were treated with a placebo.The animals were slaughtered 6 d after drug withdrawal. The gross and microscopic findings in the thymus of these animals were previously reported [5].

2.2. Samples

Thoracic and cervical thymus samples from each animal were collected and placed in RNAlater solution (Ambion, Thermo Fisher Scientific, Waltham, MA) to preserve the RNA integrity for molecular investigation.

2.3. Total RNA extraction and quantitative PCR

The expression of c-Myc, TCF3, TP63, KRT5, BAX and BCL2 in the thoracic and cervical thymus was investigated by quantitative PCR (qPCR). For this purpose, fifty milligrams of thymus were disrupted using a TissueLyser II (Qiagen, Hilden, Germany) with stainless steel beads in 1 mL of TRIzol (Invitrogen, Thermo Fisher Scientific). Total RNA was purified from any residual genomic DNA with a DNA-free kit (Ambion). The integrity of the RNA was confirmed by the Experion Automated Electrophoresis Station (Bio-Rad, Hercules, CA), and the concentration was measured by a spectrophotometry. cDNA was synthesised from 1 µg of total RNA using ImProm-II reverse transcriptase (Promega, Madison, WI) and random primers (Promega). To determine the amount of the specific target genes, cDNA was subjected to qPCR using the SYBRGreen method and the IQ5 (Bio-Rad) detection system. The primer sequences were designed using Primer Express v 1.5 (Applied Biosystems, Thermo Fisher Scientific) (Table 1). The peptidylprolyl isomerase A (cyclophilin A, PPIA) gene was used as a housekeeping control gene, as previously reported [14]. The expression level of each target gene was calculated using the $2^{-\Delta Cq}$ method, where $\Delta Cq = Cq_{\text{target gene}} - Cq_{\text{housekeeping gene}}$ [15].

2.4. Statistical analysis

The data were analyzed using GraphPad InStat version 3.00 (GraphPad Inc., San Diego, CA). The analysis of c-Myc, TCF3, TP63, KRT5, BAX and BCL2 gene expression and the analysis of the ratio of BAX and BCL2 expression was performed using one-way analysis of variance (ANOVA), followed by Dunnett's post hoc-test versus the control group K. If Bartlett's test suggested that the difference between the standard deviations of each group was significant, then the nonparametric Kruskal-Wallis test with Dunn's post-test versus the control group was applied. The Grubbs test was used to reveal potential outliers. A P value of < 0.05 was considered statistically significant. The data are shown as the mean arbitrary units ($2^{-\Delta Cq}$) ± SEM.

3. Results

In the cervical thymus, DEX administration (group A) increased c-Myc expression (mean of mRNA arbitrary units ± SEM: $9.46 \times 10^{-2} \pm 1.49 \times 10^{-2}$) compared with the control group K ($4.70 \times 10^{-2} \pm 3.02 \times 10^{-3}$) ($P < 0.01$) (Fig. 1a), TCF3 expression ($8.74 \times 10^{-3} \pm 4.54 \times 10^{-3}$) compared with the control group K ($8.48 \times 10^{-4} \pm 9.88 \times 10^{-5}$) ($P < 0.05$) (Fig. 1b), TP63 expression ($8.64 \times 10^{-2} \pm 2.71 \times 10^{-2}$) compared with the control group K ($1.56 \times 10^{-2} \pm 1.85 \times 10^{-3}$) ($P < 0.01$) (Fig. 1c) and KRT5 expression ($2.31 \times 10^{-1} \pm 1.99 \times 10^{-2}$) compared with the control group K ($3.75 \times 10^{-2} \pm 6.20 \times 10^{-3}$) ($P < 0.01$) (Fig. 1d). Conversely, DEX administration decreased BAX expression ($1.54 \times 10^{-3} \pm 1.17 \times 10^{-4}$) compared with the control group K ($3.44 \times 10^{-3} \pm 2.38 \times 10^{-4}$) ($P < 0.01$) (Fig. 2a). No change in BCL2 expression was observed (Fig. 2b). The administration of PRD (group B) caused no change in the expression of all considered genes (Fig. 1 and 2).

In the thoracic thymus, DEX administration (group A) increased TCF3 expression ($5.68 \times 10^{-3} \pm 1.04 \times 10^{-3}$) compared with the control group K ($2.12 \times 10^{-3} \pm 2.51 \times 10^{-4}$) ($P < 0.01$) (Fig. 1b), TP63 expression ($6.87 \times 10^{-2} \pm 8.70 \times 10^{-3}$) compared with the control group K ($2.95 \times 10^{-2} \pm 3.62 \times 10^{-3}$) ($P<0.01$) (Fig. 1c) and KRT5 expression ($1.79 \times 10^{-2} \pm 6.59 \times 10^{-3}$ compared with the control group K ($5.37 \times 10^{-2} \pm 1.88 \times 10^{-3}$) ($P < 0.05$) (Fig. 1d). No change in c-Myc (Fig. 1a) or BCL2 expression was observed (Fig. 2b). Conversely, DEX administration decreased BAX expression ($3.20 \times 10^{-3} \pm 2.67 \times 10^{-4}$) compared with the control group K ($4.91 \times 10^{-3} \pm 5.35 \times 10^{-4}$) ($P < 0.05$) (Fig. 2a). The administration of PRD (group B) caused no change in the expression of all considered genes (Fig. 1 and 2). The BAX/BCL2 ratio was statistically different in both cervical and thoracic thymus of Group A compared to the controls ($P < 0.01$ and $P < 0.05$, respectively) (Fig. 2c).

4. Discussion

Council Directive 96/22/EC [1], as amended by Directives 2003/74/EC [16] and 2008/97/EC [17], stipulates that all use of steroids, β-agonists or other substances for the chemical manipulation of animal growth is severely banned in the EU. However, results from studies conducted by the Italian Health Ministry indicate that these substances are persistently used, and therefore a permanent commitment by the public veterinary services for their prevention and control is required. The thymus represents a GCs target tissue, and in vitro or ex vivo qualitative and semi-quantitative morphological investigations to identify the cellular effects of GCs were reported [2,4,18].

Indeed, the thymus weight and volume of beef cattle following long-term administration of low doses of DEX were significantly reduced compared with the controls [4]. Moreover, DEX-treated animals showed severe thymus atrophy, characterized by a serious volume

reduction of the organ, which in some animals almost disappeared and was replaced by fat tissue. Histologically, the thymic cortex undergoes atrophy, while the medullary framework was still present though reduced, showing a pronounced rarefaction of lymphocytes [5]. Conversely, no histological change was observed in the thymus following long-term treatment with PRD [5].

However, partial recovery of thymus weight and structure after 25 days followed by complete recovery after 32 days was observed in veal calves [3] and similar results have been observed in thymus of beef cattle examined 26 days after the end of treatment [4]. Thus, it is conceivable that the thymus preserves an intrinsic ability to regenerate, but the molecular mechanisms controlling the regeneration of the thymus are largely unknown. Previous work in mice demonstrated that c-Myc, TP63, and TCF3 gene expression was up-regulated in TECs during peak thymic atrophy and was down-regulated at later time points when thymuses were undergoing regeneration [6]. These transcription factors were previously shown to regulate differentiation of epithelial stem cells in various tissues [7,8,19] suggesting a role in reconstruction/maintenance of the epithelial cell network. Consistent with these findings, our results showed an up-regulation of the same transcription factors in thymus of beef cattle experimentally treated with a low doses of DEX for a long-term. In contrast, the treatment with PRD did not induce any expression changes in the genes examined in this study. Since the DEX administration induces the thymus atrophy [4], whereas PRD treatment appears to have no effects on the thymus tissue [5], the expression of the transcription factors might play a role in regeneration of the thymic stroma.

Moreover, it has been demonstrated that DEX-induced damage of the thymus resulted in proliferation of subset of TEC precursors expressing KRT5 [6]. This active expansion could explain the significantly over-expression of KRT5 observed in the atrophic thymus of DEX-treated beef cattle compared to controls.

Glucocorticoids influence the growth and differentiation of thymocytes through several mechanisms including apoptosis [20]. The apoptotic effect of GCs could shift the balance between expression of pro-survival and pro-apoptotic factors, ultimately leading to cell death or apoptosis [21]. In this study, DEX administration caused a decrease of BAX expression, whereas BCL2 expression remain unchanged. Moreover, the ratio of BAX and BCL2 expression diminished. These results suggest that an anti-apoptotic effect is occurring through the mitochondrial pathway, and this may support the hypothesis that regeneration activity occurs in the thymus parenchyma 6 days after the treatment is discontinued.

Thus, our data suggested that the observed recovery of thymus of DEX-treated beef cattle might be mediated by several events, including the elevated expression of transcription factors involved in differentiation of epithelial stem cells, active proliferation of TECs subset and inhibition of apoptosis. The expression of this panel of genes appears characteristic of the animals treated with DEX and not of PRD-treated animals. Indeed, previous studies showed the inability of PRD to induce macro- and microscopical lesions (i.e. atrophy) in thymus. Therefore, it is not surprising that PRD does not induce alteration of genes involved in regeneration pathway.

Acknowledgments

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Declarations of interest: none.

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307 **Table 1** Primer sequences used for qPCR.

Gene	Sense	Sequences 5' → 3'	Amplicon size (bp)	GeneBank accession number
c-Myc	Forward	ATGCCACGTGTCTACCCATCA	100	NM_001046074
	Reverse	GACCCTGCCACTGTCCAACT		
TCF3	Forward	TGGCTGAGTGCACCCTGAA	107	XM_002691408
	Reverse	CCGGGCCAATTCGTAGTACTT		
TP63	Forward	TTCCGTGAGCCAGCTTATCA	100	NM_001191337
	Reverse	GTGGGTGCCCATCATAGGAAT		
KRT5	Forward	GAGCCTTTGTTAGAGCAGTACATCAA	100	NM_001008663
	Reverse	CCTGCATATTCCTGAGCTCTGA		
BAX	Forward	AAGCGCATCGGAGATGAATT	100	NM_173894
	Reverse	CCGCCACTCGGAAAAAGAC		
BCL2	Forward	TGGTGGGCGCTTGCAT	100	NM_001166486
	Reverse	TTCTGCTGCTTCTTGAATCTTCTG		
PPIA	Forward	GCCCCAACACAAATGGTT	95	NM_178320
	Reverse	CCCTCTTTCACCTTGCCAAAG		

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Figure captions

Fig. 1. Effects of dexamethasone-21-sodium phosphate (DEX; group A) or prednisolone (PRD; group B) on c-Myc (a), TCF3 (b), TP63 (c) and KRT5 (d) gene expression compared with the control group K in the cervical and thoracic thymus of beef cattle. The results are presented as the means ± SEM. The y-axes show arbitrary units representing relative mRNA expression levels. *$P < 0.05$, **$P < 0.01$ versus the control group K.

Fig. 2. Effects of dexamethasone-21-sodium phosphate (DEX; group A) or prednisolone (PRD; group B) on BAX (a) and BCL2 (b) gene expression compared with the control group K in the cervical and thoracic thymus of beef cattle. The results are presented as the means ± SEM. The y-axes show arbitrary units representing relative mRNA expression levels. Effects of dexamethasone-21-sodium phosphate (DEX; group A) or prednisolone (PRD; group B) on ratio of BAX and BCL2 expression (c) in cervical and thoracic thymus of beef cattle. *$P < 0.05$, **$P < 0.01$ versus the control group K.